Imaging the Function of Gene Products

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The recent completion of DNA sequencing projects for human and mouse genomes has shifted the focus of molecular research from discovery of new genes to interrogating regulation and function of proteins and signaling pathways. Because of the ready availability of DNA microarrays, a large number of published studies have analyzed patterns of gene expression in normal physiology and in response to a variety of different disease processes. However, there are multiple examples that show discordance among levels of gene expression, amounts of total protein, or actual functions of proteins. Regulation of signaling pathways commonly is accomplished through post-translational modifications of proteins, such as phosphorylation or ubiquitination, and/or alterations in subcellular compartmentation. These data emphasize that interrogating functions of proteins is essential for understanding normal physiology, perturbations produced by disease, and effects of new molecularly-targeted therapies.

To analyze proteins in the integrated physiology of a living organism, there is an ongoing effort to develop imaging strategies and probes to monitor protein function in living cells and intact animals. Currently, these molecular imaging approaches have been used almost exclusively in isolated cells or mouse models of disease. Studies performed in these model systems demonstrate that real-time imaging of spatial and temporal changes in protein function provides data about pathogenesis of disease and response to therapy that would be difficult to obtain with other methods. The exciting data generated in cells and animal models also provides the impetus to translate these imaging technologies to applications in humans. In the following sections, we review applications of molecular imaging to analyze function and regulation of proteins.

1. Protein-protein interactions

Formation and dissociation of protein complexes regulate many essential processes within cells, including cell cycle progression, signal transduction pathways, and cell motility. Abnormal regulation of protein-protein interactions occurs commonly in disease processes such as cancer and autoimmunity. Because of the significance of protein interactions to normal biology and disease, there also is increased emphasis on exploiting protein interactions as molecular targets for therapy. A number of techniques have been developed to identify and characterize protein interactions in cultured cells. However, it only recently has become possible to image protein interactions in living animals, thereby providing the potential to better understand the regulation of protein interactions in vivo.

The two-hybrid assay is one of the best-characterized methods for probing protein interactions in vitro. This technique can be used to screen for new interacting proteins or investigate interactions between two specific proteins. Two-hybrid assays are based upon the fact that many transcription factors are comprised of distinct domains for recognizing DNA sequences and activating transcription. In its simplest form, a two-hybrid assay is performed by fusing one protein of interest to a DNA binding domain and a second protein of interest to an activation domain for transcription. In the presence of interacting proteins, the hybrid transcription factor is reconstituted to drive expression of a reporter gene.

Researchers in molecular imaging have adapted the two-hybrid assay for use with radiotracer and optical techniques. Luker et al used PET imaging to detect and quantify the well-characterized interaction between the tumor suppressor p53 and large T antigen from SV40 virus. Co-expression of these proteins fused to a DNA binding domain or an activation domain, respectively, could be detected in tumor xenografts using Herpes simplex virus thymidine kinase as a reporter gene and the radiolabeled substrate 9-(4-[^{18}F]-Fluoro-3-

hydroxymethylbutyl)guanine (^{18}F-FHBG) (1). Accumulation of radiotracer in response to interacting proteins was significantly greater than non-interacting control proteins, and signals produced by interacting proteins were proportional to relative amounts and duration of protein interactions (2). A similar approach was used by Ray et al to monitor interactions between the helix-loop-helix nuclear proteins MyoD and Id, two molecules that regulate differentiation of skeletal muscle (3). Binding of hybrid MyoD to hybrid Id activated expression of firefly luciferase, thereby enabling the magnitude and duration of interactions between these two proteins to be detected and quantified in vivo with bioluminescence imaging. Collectively, these studies suggest that the two-hybrid strategy is a generalizable method to detect protein interactions in living animals and potentially monitor efficacy of new pharmaceutical compounds targeted to protein interactions.

Although two-hybrid systems have been used successfully to analyze protein interactions in mouse models, there are important limitations to using this method to study spatial and temporal regulation of interacting proteins. Because the two-hybrid system relies upon transcription of a reporter gene to monitor interacting proteins, it is essential that the proteins of interest must associate in the nucleus. The two-hybrid system lacks good temporal resolution because there is a delay between association and dissociation of target proteins and changes in amounts of reporter proteins. Finally, it is difficult to study interactions of transcription factors with other proteins because a transcription factor may activate expression of the reporter gene independent of protein interactions.

To overcome limitations of the two-hybrid system, several alternative methods have been developed to monitor interacting proteins in cultured cells. Recently, investigators have adapted the protein fragment complementation technique for quantifying protein interactions in living animals. Protein fragment complementation is based upon dividing a monomeric enzyme into two separate fragments that do not function independently or reassemble spontaneously to function. Activity of the divided enzyme is reconstituted only when the fragments are brought together by interacting proteins. Protein fragment complementation has been accomplished with firefly and Renilla luciferases, thereby allowing protein interactions to be detected and quantified with bioluminescence imaging. For example, Paulmurugan et al divided firefly luciferase into N- and C-terminal domains, based on the crystal structure of the enzyme (4). Although each domain had residual activity, interaction between these domains mediated by MyoD and Id produced greater bioluminescence than either domain alone. Luker et al used a library screening approach to identify two complementing fragments of firefly luciferase (5). Only the N-terminal fragment had residual activity, and interactions between several different pairs of interacting proteins reconstituted significantly greater luciferase activity than observed in the absence of interacting proteins. This system also was used to measure pharmacologic regulation of protein interactions in vivo, suggesting that these systems can be used to aid drug development. Comparable enzyme complementation pairs also have been derived and validated for Renilla luciferase (6). In principle, combined used of firefly and Renilla complementation could allow two different pairs of interacting proteins to be monitored in the same group of cells.

Although MR imaging of protein interactions has not been accomplished in living animals, Perez et al developed biocompatible nanoparticles to detect molecular interactions (7). When attached to interacting proteins, conversion of these particles from disperse solutions to stable assemblies could be detected with magnetic resonance imaging techniques. Potentially, this system could be developed into an assay for protein interactions for use in intact cells or living animals. Overall, these data obtained with several different imaging techniques confirm that protein interactions can be monitored in living animals and emphasize the potential for these tools to advance studies of disease and improve development of new therapeutic compounds.

2. Protein degradation

Stability of proteins within a cell is a key determinant of protein function, and regulated degradation of key proteins is essential for controlling signaling pathways in cell growth and division, apoptosis, and inflammation. The primary regulator of protein stability and degradation in cells is the proteasome, an enzyme complex comprised of multiple catalytic and modulating subunits. With rare exceptions, proteins are marked for degradation in the proteasome by attachment of chains of 4 or more ubiquitin proteins, thereby conferring recognition of target proteins by the proteasome. Derangements in function of the ubiquitin-proteasome system are associated with diseases including cancer, stroke, and neurodegeneration. Pharmacologic blockade of the proteasome has been shown to retard growth of several different types of tumors, as evidenced by the therapeutic success of the proteasome inhibitor bortezomib in patients with multiple myeloma and in clinical trials of other malignancies (8,9). Collectively, these data emphasize the importance of the ubiquitin-proteasome system in health and disease.

To monitor function of the proteasome in living animals, Luker et al developed a genetically-encoded reporter for bioluminescence imaging of proteasome function and inhibition (10). The reporter consisted of firefly luciferase fused with 4 copies of ubiquitin to target the reporter for degradation in the proteasome. Under baseline conditions, more than 90% of newly synthesized ubiquitin-firefly luciferase reporter was degraded within 15 minutes, while more than 75% of firefly luciferase remained after 4 hours. The markedly decreased half-life of the ubiquitin-firefly luciferase fusion protein resulted in this reporter producing almost 2-logs less bioluminescence than firefly luciferase in cultured cells and tumor xenografts. In mice bearing tumor xenografts of cells expressing the ubiquitin-firefly luciferase reporter, treatment with the clinically-approved proteasome inhibitor bortezomib prevented degradation of the reporter and increased bioluminescence to levels observed with firefly luciferase. Collectively, this system provided a repetitive, in vivo assay for proteasome function, thereby facilitating testing and validation of proteasome inhibitors and studies of the proteasome in disease.

Genetically-encoded reporters for degradation of specific substrates of the proteasome also have been developed in a similar manner. For example, Zhang et al fused firefly luciferase to p27, a key negative regulator of the cell cycle (11). The p27-firefly luciferase reporter was stabilized in response to drugs that blocked cell proliferation or inhibition of the signal to degrade p27, thereby reproducing the expected dynamics of endogenous p27 during the cell cycle. Similarly, Gross et al used a fusion between firefly luciferase and the protein IκBα to monitor degradation of this protein as part of the signaling pathway that activates NFκB, an important regulator of inflammation and malignancy (12). Degradation of IκBα-firefly luciferase occurred in response to known stimuli that activate NFκB, and the reporter was stabilized after inhibition of the proteasome. For both studies, changes in stability of both proteins corresponded to alterations in bioluminescence that could be detected with in vivo imaging. Collectively, these studies suggest that comparable fusion proteins could be made to monitor the proteasome and specific substrates for degradation.

In addition to bioluminescence imaging, PET imaging has been used to monitor degradation of a substrate for the proteasome, thereby providing an indirect assay for proteasome inhibition. The oncogene Her2, an important regulator of breast cancer progression, is protected from degradation in the proteasome by the heat shock chaperone protein Hsp90. Smith-Jones et al labeled a F(ab')2 fragment of the clinically-used anti-Her2 antibody Trastuzumab with the PET isotope Ga^{68} to develop an imaging probe for Her2 (13). Treatment with an inhibitor of Hsp90 caused degradation of Her2, which could be detected by PET imaging as a loss of signal from the Her2 probe. In principle, this imaging probe could be translated to humans to monitor proteasome function indirectly and guide drug therapy, and similar approaches could be used to monitor other cell surface receptors that are substrates for the proteasome.

3. Enzyme activity

Similar to protein interactions and protein degradation, intracellular and extracellular proteases control a wide variety of biologic processes and may be regulated abnormally in diseases including cancer, cardiovascular disease, arthritis, and infectious disease. Proteases are regulated predominantly at post-translational levels, such as changes in compartmentalization of enzyme or substrate or transition from a pro-enzyme to a functionally active form. Therefore, it is essential to interrogate proteases with molecular imaging strategies that monitor enzymatic activity of proteases, rather than assaying changes in gene expression. In this section, we present examples of imaging probes and methods to detect and quantify activity of specific proteases and enzymes in vivo.

Molecular imaging probes for proteases exploit the fact that different enzymes cleave target substrate proteins at specific recognition motifs. These recognition motifs can be incorporated into reporter molecules to allow imaging of protease function. Researchers in the Center for Molecular Imaging Research at Harvard have developed a number of near-infrared fluorescence (NIRF) probes for detecting protease activity in vivo with optical imaging (14). These probes are based on polymers or peptides that incorporate NIRF molecules separated by consensus cleavage sites for a protease of interest. In the intact imaging probe, close spatial proximity of NIRF molecules, such as Cy5.5, produces self-quenching to limit emission of light. Cleavage at the recognition motif separates the dye molecules, resulting in increased fluorescence as a reporter of protease activity. For example, this imaging platform has been used to interrogate function and pharmacologic inhibition of matrix metalloproteinases, thrombin, and various lysosomal cathepsin proteases (15-17). These proteases impact development, progression, and treatment of medically important diseases including cancer, atherosclerosis, and arthritis, making these and related imaging probes key reagents for investigating effects of targeted therapies.

There also is ongoing research to develop MR probes for detecting proteases and other enzymes. Zhao et al developed magnetic resonance sensors for detecting protease activity based on interaction of a biotinylated substrate with a magnetic nanoparticle linked to the binding partner avidin (18). In the absence of active protease, the substrate molecules cluster and produce high T2 relaxivity. Cleavage of the target substrate relieves clustering of the magnetic nanoparticles to produce a change in T2 signal that can be detected with MR imaging. While this strategy works efficiently for proteases derived from cell lysates, translating this technique to intact cells or animals will be challenging technically. MR imaging has been used successfully to image function of β-galactosidase, a commonly used reporter gene (19). The imaging agent used galactopyranose, a substrate for β-galactosidase, to block access of water molecules to the first coordination sphere of a gadolinium ion. Cleavage of the blocking substrate changed the T1 signal, thereby allowing detection of active β-galactosidase after intracellular injection of the substrate. Subsequent research by this laboratory has produced a cell permeable MR contrast agent to translocate gadolinium into cells (20), suggesting that it may become possible to use MR reporters to detect intracellular proteases and enzymes.

4. Apoptosis

Apoptosis is a form of programmed cell death that is essential for normal development and maintenance of tissue homeostasis. Extensive research using gain- or loss-of function models has demonstrated that abnormal regulation of apoptosis can be a primary cause of multiple diseases. For example, cancer cells commonly have defects in initiating apoptosis or overexpress molecules that block apoptosis, thereby allowing malignant cells to escape normal control by signals in the tissue microenvironment. Apoptosis is a common effector pathway activated by many chemotherapeutic agents to exert cytotoxic effects on malignant cells and limit growth of tumors. In addition, excessive cell death mediated by apoptosis is associated with diseases including neurodegeneration, stroke, and ischemic heart disease.

One of the defining biochemical events in apoptosis is activation of caspases, a family of intracellular proteases that are categorized based on their functions as “initiator” or “effector” molecules in this cell death pathway. Caspases cleave substrate proteins that contain defined amino acid recognition motifs that are specific for particular members of this family of enzymes. These conserved recognition motifs form the basis for molecular imaging of caspase function. For example, Messerli et al designed a NIRF imaging probe for caspase 1, a caspase that may initiate apoptosis in response to some signals and also functions to promote inflammation through production of interleukins (21). The NIRF probe for caspase 1 was based on a self-quenched fluorescent molecule that incorporated a peptide substrate for this protease (amino acids YVAD). Cleavage of the NIRF probe by activated caspase 1 relieved self-quenching by the NIRF dye molecules and produced a fluorescence signal. This probe allowed specific detection of caspase 1 in cultured cells and in tumor xenografts, using fluorescence reflectance imaging to detect and quantify the active protease in real time.

Caspase 3, a key effector molecule in execution of apoptosis in cells, also has been assayed in vivo with molecular imaging techniques. Laxman et al devised a novel reporter for protease activity based on fusion of the estrogen receptor (ER) to the N- and C-termini of firefly luciferase (22). The ER domains were separated from firefly luciferase by recognition motifs for caspase 3 (DEVD). Function of firefly luciferase was attenuated by the presence of both ER domains under baseline conditions, while cleavage at DEVD sites by activated caspase 3 removed the ER silencing domains and markedly increased bioluminescence. Tumor cells stably expressing this caspase 3 reporter were implanted as tumor xenografts in nude mice. After treatment of mice with TRAIL to induce apoptosis in tumor cells, luciferase activity increased by approximately 3-fold as quantified by bioluminescence imaging, thereby providing an in vivo assay for therapy-induced apoptosis. Furthermore, this system could be used to detect apoptosis under different experimental conditions or be adapted readily to detect other intracellular proteases.

As an alternative approach to image apoptosis, investigators have focused on developing probes for the membrane lipid phosphatidylserine. As an early event in apoptosis, a phospholipid scramblase translocates phosphatidylserine from the inner to outer leaflet of the cell membrane, thereby exposing this lipid to the extracellular space. Imaging agents that incorporate annexin V, a molecule that binds specifically to phosphatidylserine, have been used to detect apoptosis in vivo. For example, annexin V has been radiolabeled with Tc^{99m}, F^{18}, or I^{124} for single-photon or PET radiotracer imaging of apoptotic cell death in cardiac allograft rejection or immune-mediated death of hepatocytes (23,24) (25). More recently, Josephson and colleagues developed and validated a fluorescent, magnetic nanoparticle conjugated to annexin V (26). The annexin V nanoparticle accumulated in ischemic myocardium, as evidenced by significantly lower T2* values on in vivo MR imaging and fluorescence analysis of excised tissue specimens (27). Potentially, the magnetic annexin V nanoparticle will overcome some limitations of nuclear imaging of apoptosis, including limited spatial resolution and the inability to acquire images of cardiac wall motion. However, it is important to note annexin V imaging does not necessarily differentiate apoptotic from necrotic cells because disruption of the cell membrane in necrotic cells will allow annexin V molecules to bind phosphatidylserine that remains on the inner leaflet.

5. Summary

As emphasized by the NIH Roadmap initiatives, there is increased emphasis on development and application of imaging techniques to interrogate normal and pathologic physiology in vivo. The probes and imaging strategies described above are representative of ongoing research efforts to advance our ability to interrogate cellular and molecular functions of proteins in animal models and ultimately in people. It is expected that continued improvements

in the chemistry and biology of imaging probes and physics of instrumentation will allow molecular imaging to greatly improve diagnosis and treatment of a variety of disease processes.

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